

Genetics and animal models of familial pulmonary fibrosis

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Abstract

Pulmonary fibrosis is caused by the interplay between genetic and environmental factors. Recent studies have revealed various genes associated with idiopathic pulmonary fibrosis, as well as the causative genes for familial pulmonary fibrosis. Although increased death or dysfunction of type 2 alveolar epithelial (AT2) cells has been detected in lung specimens from pulmonary fibrosis patients, it remains unclear whether and how AT2 cell death or dysfunction is responsible for the progression of pulmonary fibrosis. A recent study showed that increased AT2 cell necroptosis is the initial event in pulmonary fibrosis by analyzing patients with familial pulmonary fibrosis and an animal model that harbors the same mutation as patients. The contribution of AT2 cell necroptosis to the pathogenesis of pulmonary fibrosis has not been identified in animal model studies, which validates the effectiveness of genetic analysis of familial diseases to uncover unknown pathogenesises. Thus, further extensive genetic studies of pulmonary fibrosis along with functional studies based on genetic analysis will be crucial not only in elucidating the precise disease process but also, ultimately, in identifying novel treatment strategies for both familial and non-familial pulmonary fibrosis.

Keywords: alveolar epithelial cells, necroptosis, surfactant

Introduction

Immune-mediated diseases are caused by a complex interplay between environmental and genetic factors. Genome-wide association studies have revealed a variety of gene loci that are associated with immune-mediated diseases, and many immunological studies in animal models have revealed the principles of the immune system (1–3). The molecular basis of most immune-mediated diseases, however, remains unclear (4). Several recent genetic analyses of familial immune-mediated diseases successfully identified single causative mutations (3, 5, 6). These studies revealed the function of each gene in humans, illustrating that the specific symptoms or laboratory data of an individual patient can provide crucial insights into basic physiological mechanisms. However, debates sometimes arise regarding whether these studies lead to improved understanding of sporadically occurring diseases that have similar symptoms. Indeed, the rare mutations identified in familial diseases are rarely found in related diseases without any familial connection. This finding raises a broader question about what we can learn from studies of

causative genes in rare immune-mediated diseases to understand human diseases and develop therapeutic strategies for treating both specific genetic diseases and related diseases. To address this question, this review discusses how studies of causative mutations in familial diseases will help pave the way for understanding not only specific familial diseases but also unappreciated disease progression mechanisms that have not been identified in animal models, such as those of pulmonary fibrosis.

Non-familial pulmonary fibrosis

Pulmonary fibrosis is caused by a variety of factors, and some types of pulmonary fibrosis are reversible (7, 8). For example, drug-induced pulmonary fibrosis can be reversed by early-phase treatment, while idiopathic pulmonary fibrosis (IPF) is progressive, although pirfenidone and nintedanib can prolong the time to disease progression, resulting in prolonged survival (7, 9). IPF is the most common clinical form

of interstitial lung disease, and the median survival time is 3–5 years after diagnosis. Hereafter, this review will focus on IPF as an example of an interstitial lung disease because reviewing all types of interstitial lung disease is not feasible within the page limits. Histologically, IPF is characterized by the presence of usual interstitial pneumonia with fibroblastic foci. The progression rates of fibrosis vary among IPF patients, with some showing slow progression and others showing acute exacerbations resulting in rapid declines in lung function (7, 9). Many studies have identified candidate molecules or pathways underlying IPF pathology, including alveolar epithelial cell (AEC) death, epithelial regeneration, macrophage activation and fibroblast differentiation (7). However, the cause of IPF is still unknown, as indicated by the word “idiopathic” in the name.

AECs are crucial in pulmonary fibrosis

Pulmonary fibrosis involves multiple cell types, including interstitial macrophages, fibroblasts and AECs [alveolar type 1 (AT1) and type 2 (AT2)], which maintain the alveolar surface tension required for proper gas exchange. Although macrophages and fibroblasts play crucial roles in pulmonary fibrosis, the literature on these cells is not included in this review, which focuses only on AECs. The initial event in pulmonary fibrosis has been thought to occur in AECs. AT2 cells are a heterogeneous population with regenerative and secretory capacity that are located in the alveoli (8, 10). Furthermore, AT2 cells have two major functions. (i) AT2 cells facilitate efficient gas exchange by the production and secretion of surfactant and the transepithelial movement of water and ions. The pulmonary surfactant that lines alveoli is essential for lowering alveolar surface tension, which prevents atelectasis at the end of expiration. AT2 cells synthesize surfactants, store them in lamellar bodies and secrete them into the alveolar lumen; this process is required for lowering alveolar surface tension. AT2 cells transport sodium from the apical surface into the interstitium through epithelial sodium channels and express Na/K ATPase to maintain intracellular Na and K concentrations. (ii) AT2 cells regenerate the alveolar epithelium after injury by serving as progenitor cells. AT2 cells are capable of self-renewal and can function as progenitor cells for AT1 and AT2 cells (11). In response to lung injury and stress, AT2 cells proliferate, and some of them differentiate into AT1 cells, which helps to organize normal alveolar structures because AT1 cells are involved in barrier function and gas exchange. Various signaling pathways associated with AT2 cell proliferation and the AT2-to-AT1 transition have been reported but require further elucidation.

The genetics of IPF

Genome-wide linkage and association studies have identified common genetic variants associated with IPF risk, including genes associated with mucus secretion (*MUC5B*), immune function (*IL1RN*, *IL8*, *TLR3* and *TGFB1*), epithelial barrier function (*DSP* and *DPP9*), telomeres (*TERT*, *TERC*, *OBFC1*, *TINF2*, *DKC1*, *RTEL1* and *PARN*), surfactants (*SFTPC*, *SFTPA2* and *ABCA3*) and cell cycle regulation (*CDKN1A*, *KIF15* and *MAD1L1*) (7, 12). The rs35705950 variant in the

promoter region of *MUC5B* is associated with an ~7-fold increase in IPF risk (13, 14). Multiple subsequent studies demonstrated the increased risk of IPF associated with this variant (13, 15, 16); thus, this variant is currently the most significant genetic risk factor for IPF. Although the mechanism by which the *MUC5B* promoter variant is associated with pulmonary fibrosis remains unclear, one paper demonstrated that *Muc5b* over-expression impairs mucociliary clearance, which is associated with the extent and persistence of bleomycin-induced pulmonary fibrosis (17). In addition, *Muc5b*-deficient mice developed bronchial hyperplasia, interstitial thickening, alveolar collapse, immune cell infiltration, disorganized elastin fibers and collagen deposition, indicating essential roles of *MUC5B* in normal lung function (18).

Familial pulmonary fibrosis

The causative genes associated with familial pulmonary fibrosis are mainly divided into telomere- and surfactant-related genes.

Telomere-related genes

The telomerase complex catalyzes the addition of repeated DNA sequences to the telomere region. Previous studies have revealed heterozygous mutations in the *TERT* and *TERC* genes in patients with familial pulmonary fibrosis and sporadic IPF (19–21). The *TERT* gene encodes telomerase reverse transcriptase, which forms the telomere complex, together with the transcript of the telomerase RNA component (*TERC*), which is needed to maintain telomere length. Mutations in the telomerase-related genes *DKC1*, *NAF1*, *RTEL1*, *PARN*, *TINF2* and *ZCCHC8* (21–25) have been reported and are much rarer than mutations in *TERT* and *TERC*. Functional analyses of mouse models are summarized in Table 1. The short telomeres in AT2 cells preferentially lead to cellular senescence; these cell cycle alterations are mediated by p53 and p21 (26, 27). AT2 cell-specific deletion of *TERT* in mice did not spontaneously result in pulmonary fibrosis but induced lung injury, inflammation and fibrosis upon bleomycin administration. This selective *Tert* deficiency induced cellular senescence in AT2 cells (28). Hypomorphic *Dkc1* mutant mice show disruption in the normal architecture of the lung parenchyma but do not develop pulmonary fibrosis (29). Pathological changes in the lungs of mice deficient in *Naf1* (30), *Rtel* (31), *Parn* (32), *Tinf2* (33) or *ZcchC8* (25) have not been reported. Although introducing a mutation observed in human patients into a mouse gene does not always lead to similar symptoms in mice, if lung phenotypes are present, these mouse models would be valuable in understanding the molecular networks that cause pulmonary fibrosis.

Surfactant-related genes

Pulmonary surfactant is a phospholipid-rich substance that is produced in the distal airway and alveoli. These phospholipids are ~70–80% phosphatidylcholine, and dipalmitoyl phosphatidylcholine constitutes the surface-active component. Approximately 10% of surfactants are neutral lipids, including cholesterol, and ~10% of surfactant molecules form specific proteins that are produced by AT2 cells and

Table 1. Causative genes of familial pulmonary fibrosis and animal models

Causative gene	Mouse model	Phenotypes of mouse model	References about mouse models
<i>SFTPA1</i>	Knockin mouse	Pulmonary fibrosis	(40)
<i>SFTPA1</i>	Knockout mouse	Increase of pro-inflammatory cytokines and increased mortality and collagen deposition after induction of bleomycin	(47)
<i>SFTPA2</i>	Mice do not have the gene		
<i>SFTPC</i>	Knockin mouse	Early influx of pro-inflammatory CCR2 ⁺ Ly6C ^{hi} immature macrophages	(39)
<i>TERT</i>	Knockout mouse (AT2-specific)	(1) No spontaneous development of pulmonary fibrosis (2) Lung injury, inflammation and fibrosis by bleomycin treatment	(28)
<i>TERC</i>	Knockout mouse	Elevation in various pro-inflammatory cytokines in the lungs	(27)
<i>DKC1</i>	Hypomorphic mutant	Disruption of the normal architecture of the lung parenchyma	(29)
<i>NAF1</i>	Knockout mouse	<i>Naf1</i> ^{+/-} mice had no abnormalities in major organs	(30)
<i>RTEL1</i>	Knockout mouse	No description	(31)
<i>PARN</i>	Knockout mouse	Embryonic lethality	(32)
<i>TINF2</i>	Knockout mouse	Embryonic lethality	(33)
<i>ZCCHC8</i>	Knockout mouse	No description about lung pathology	(25)

terminal airway secretory cells. Surfactants play crucial roles in preventing alveolar collapse and in host defense. There are four surfactant proteins, known as surfactant proteins A (SP-A), B (SP-B), C (SP-C) and D (SP-D). The hydrophilic proteins SP-A and SP-D are involved in opsonizing common bacterial and viral pathogens and enhancing phagocytic killing by innate immune cells, including macrophages and neutrophils. The hydrophobic proteins SP-B and SP-C facilitate rapid adsorption of phospholipids and are required for maintaining the surfactant film during the respiratory cycle. Regarding the association of surfactants with IPF, compared to healthy patients, IPF patients exhibit reduced SP-A in bronchoalveolar lavage fluid (34), and SP-A levels are inversely correlated with the survival of IPF patients (35).

Mutations in the genes for SP-C (*SFTPC*) have been described in association with familial lung fibrosis (36). Mutations in the C-terminal BRICHOS domain of *SFTPC* increase endoplasmic reticulum (ER) stress, resulting in activation of the unfolded protein response in AT2 cells (37). ER stress leads to epithelial-to-mesenchymal transition in lung epithelial cells (38). How do *SFTPC* mutations cause pulmonary fibrosis? A recent report demonstrated that mice harboring a certain mutation at position 73 (I73T) in *SFTPC*, which is frequently detected in IPF patients, exhibited early influx of pro-inflammatory CCR2⁺Ly6C^{hi} immature macrophages (39), although it is still not known how AT2 cell dysfunction facilitates the recruitment of pro-inflammatory macrophages.

We reported a homozygous missense mutation in *SFTPA1* in one Japanese family suffering from pulmonary fibrosis (40). The mutation does not affect the expression of *SFTPA1* but disturbs the secretion of the *SFTPA1* protein. Mice harboring the same mutation in the murine *Sftpa1* gene (*Sftpa1*-KI mice) developed pulmonary fibrosis, and ~30% of the mice died by 40 weeks of age. The AT2 cells in *Sftpa1*-KI mice were prone to death by necroptosis, which is supported by the finding that *Sftpa1*-KI mice lacking *Mkl1*, an essential necroptosis gene, do not develop pulmonary fibrosis. The increase in necroptosis was caused by ER stress in AT2 cells. ER stress induces the expression of RIPK3, an activator of MLKL, thus increasing the sensitivity of AT2 cells to necroptosis. Lung specimens from a patient with the mutation showed enhanced levels of phosphorylated MLKL in AT2 cells. Thus, these data demonstrate

that enhanced AT2 cell necroptosis is the initial event that leads to pulmonary fibrosis. Although studies have yet to identify the detailed mechanism by which increased necroptosis of AT2 cells causes pulmonary fibrosis, abundant release of damage-associated molecular patterns from dying cells is likely responsible for the initial pulmonary inflammation. Previous reports demonstrated that IPF lung tissue exhibits AEC death, and several papers reported increased apoptosis or enhanced expression of proapoptotic genes in AT2 cells (41–44). Furthermore, histological analysis of IPF lung tissue also demonstrated increased AT2 cell necrosis (45). The contribution of AT2 cell death to pulmonary fibrosis was also supported by the fact that diphtheria toxin-mediated AT2 cell death was sufficient to induce pulmonary fibrosis in mice (46). However, it remains unclear whether increased AT2 cell death is truly involved in pulmonary fibrosis in human patients, owing to a lack of functional evidence that increased AT2 cell death causes pulmonary fibrosis in humans. Thus, the direct association between the *SFTPA1* mutation and increased cell death provides strong evidence that increased AT2 cell death, at least through the necroptosis pathway, is able to cause pulmonary fibrosis. Accordingly, future studies should analyze whether patients suffering from IPF or other types of interstitial pulmonary fibrosis exhibit increased AT2 cell necroptosis. In addition, although various studies have shown increased apoptosis in AT2 cells in lung specimens of pulmonary fibrosis patients (44), it remains unclear how apoptosis of AT2 cells leads to pulmonary fibrosis. The relevance of apoptosis to pulmonary fibrosis will be clearer if pulmonary fibrosis patients can be found in whom AT2 apoptosis is impaired by a single gene mutation.

Other reports have suggested that SP-A functions in immune responses. *Sftpa1*-deficient mice showed impaired macrophage phagocytosis, an increase in pro-inflammatory cytokines (47) and increased mortality and collagen deposition after the induction of bleomycin-induced pulmonary fibrosis (48). These data suggest a link between increased pro-inflammatory cytokines in the absence of SP-A and the development of pulmonary fibrosis. However, in our studies, we could not eliminate pulmonary fibrosis in *Sftpa1*-KI mice by adding SP-A (unpublished observation), indicating that pulmonary fibrosis in *Sftpa1*-KI mice is not attributable to low levels of SP-A in alveoli.

Perspectives and concluding remarks

Identifying the causative genes for familial immunological diseases has greatly helped researchers understand the roles of these genes in the human immune system. These studies cover many areas of immunology, including immunodeficiency, autoimmunity and autoinflammation. The identification of key genes will not only help us achieve a better understanding of specific familial diseases but also allow us to develop novel therapeutics to treat these diseases. What can we learn from research on causative genes in rare immune-mediated diseases in terms of understanding the human immune system? The relationship between one gene mutation and individual or various symptoms reveals the function of the gene in the human immune system, which sometimes allows us to identify unknown functions of the gene products through animal studies. The best example is the discovery that *IL2RG* mutations cause severe X-linked combined immunodeficiency that is characterized by the absence of T and natural killer (NK) cells and the presence of non-functional B cells (49). As T and NK cells developed normally in *Il2*-deficient mice (50) and *IL2*-deficient patients (51), *IL-2* receptor- γ (*IL-2R γ*), which is encoded by *IL2RG*, was suggested to be shared with other cytokine receptors through the discovery that *IL2RG* was the causative gene in severe X-linked combined immunodeficiency. Subsequent studies demonstrated that *IL-2R γ* is a shared receptor component for *IL-2*, *IL-4*, *IL-7*, *IL-9*, *IL-15* and *IL-21* (52). This series of discoveries enabled a deeper understanding of cytokine signaling and the precise diagnosis of immunodeficient patients. We also found a missense homozygous mutation in *PSMB8* in patients suffering from fever, progressive partial lipodystrophy and hepatosplenomegaly with hypergammaglobulinemia (53). *PSMB8* is a component of immunoproteasomes, and neither *Psm8*-deficient mice nor gene-targeted mice deficient in other immunoproteasome components have shown any spontaneous inflammation or hypergammaglobulinemia. Although the molecular mechanism by which immunoproteasome dysfunction causes inflammation is still unknown, the discovery has paved the way for understanding the function of immunoproteasomes in humans and allowed us to reconsider the contribution of immunoproteasomes to inflammation and lipodystrophy in other human diseases.

Could pulmonary fibrosis be similarly understood? AT2 cell dysfunction in IPF patients was postulated many years ago, and increased AT2 cell apoptosis in IPF patients and the contribution of AT2 cell apoptosis in a bleomycin-induced mouse model have been reported. The discovery of the causative gene *SFTPA1* in familial pulmonary fibrosis and subsequent functional studies with *Sftpa1*-KI mice revealed that increased necroptosis was the initial event in pulmonary fibrosis. This study also demonstrated a molecular mechanism of pulmonary fibrosis that had not been identified in animal models. Furthermore, the genetic association between pulmonary fibrosis and telomere-related genes is notable, although it is still not understood how telomere length is associated with the risk of pulmonary fibrosis.

Although this review has stressed the importance of genetic analysis in understanding pulmonary fibrosis, the genetic basis of pulmonary fibrosis is still not fully understood, even in

familial pulmonary fibrosis. Future studies are needed to extensively investigate the genetic variations in various types of pulmonary fibrosis. Furthermore, future studies are warranted to better characterize the roles of each candidate gene in the fibrotic process by using animal models and newly generated technologies such as lung organoids. Of course, cellular responses, even in disease processes, might be beneficial in some sense. Thus, the distinction between the pros and cons of each response will be essential in finding effective target molecules or networks to treat pulmonary fibrosis.

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